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We evaluated whether immunization of mice with dendritic cells (DCs) loaded with 12B1 murine *bcr-abl*⁺ leukemia -derived chaperone-rich cell lysates (CRCL) induced BCR-ABL specific immune responses in vivo. We found that splenocytes from mice immunized with DCs loaded with CRCL secreted interferon γ (IFN- γ) when re-stimulated with a BCR-ABL peptide, GFKQSSKAL, indicating that BCR-ABL peptides are chaperoned by 12B1 tumor derived CRCL. Splenocytes from mice primed with 12B1 CRCL secreted higher amounts of IFN- γ and had higher cytolytic activity upon re-stimulation with 12B1 derived CRCL when compared to re-stimulation with BCR-ABL peptide alone suggesting that other antigenic peptides may also be present in the antigen repertoire of 12B1 CRCL and contribute to its superior immunogenicity. We also investigated the effects of combining imatinib with CRCL immunotherapy against 12B1. We demonstrated that the combination of imatinib with DC loaded with 12B1-derived CRCL yielded high activation of anti-12B1 specific T cells, and potent anti-tumor activity resulting in tumor-free survival in up to 63% of mice with *bcr-abl*⁺ 12B1 tumors. Our data suggest that immunotherapy can be effectively combined with imatinib for the treatment of CML.

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INTRODUCTION

With the clinical use of tumor-derived chaperone proteins as anti-cancer vaccines already in clinical trial stages, we have focused our attention on the utility of multiple chaperone protein complexes. We have utilized a free-solution-isoelectric focusing technique (FS-IEF) to obtain chaperone rich cell lysate (CRCL) from clarified tumor homogenates and have reported on its effective vaccinating potential against the murine CML model 12B1 and its effect on murine dendritic cells (DCs). We have demonstrated that the antigenicity of CRCL can be augmented further by loading it onto DCs. Moreover, published data from our laboratory indicate that CRCL effectively re-creates the danger signal to which immune cells are primed to respond augmenting the immunogenicity of bcr-abl+ apoptotic leukemia cells. To study further the immunologic potential of CRCL in murine and human CML, the following aims were proposed. 1) Examine whether murine 12B1 CML-derived CRCL elicits bcr-abl fusion peptide specific T cells 2) Study the possible in vivo synergistic effects of CRCL vaccine with imatinib mesylate a tyrosine kinase inhibitor that induces apoptosis in 12B1 bcr-abl+ cells 3) Isolate and biochemically characterize human CML-derived CRCL 4) Evaluate the effects of human CML-derived CRCL on DCs to determine if it induces maturation and/or alters DC function and examine its potential to generate CML specific CTLs.

BODY

Task 1. EXAMINE WHETHER BCR-ABL FUSION PEPTIDES ARE CHAPERONED BY 12B1 MURINE LEUKEMIA-DERIVED CRCL, AND IF THE PEPTIDES CONTRIBUTE TO CRCL'S ANTIGENICITY.

We have previously demonstrated that multiple chaperone proteins generated by free-solution iso-electric focusing (FS-IEF) elicit potent anti-tumor immunity^{1,2,3}. We have termed these as chaperone rich cell lysates (CRCL) and found that 12B1 tumor derived CRCL activated dendritic cells (DC) stimulate tumor specific T cell mediated immune responses².

We have shown that CRCL contain four major immunogenic chaperone proteins, HSP70, HSP90, gp96 and calreticulin. We further described CRCL as large, multi-member entities wherein chaperones co-separate in high molecular weight fractions despite dissociative conditions used in size exclusion chromatography³. Since chaperone proteins carry various endogenous peptides, we reasoned that tumor-derived CRCL may chaperone antigenic peptides. However, due to the isofocusing conditions used in the vaccine generation (i.e., 6 M urea), one may argue that the peptide repertoire of CRCL would be lost. To study if in fact CRCL chaperones tumor specific antigens, we generated CRCL from 12B1, a *bcr-abl* positive murine leukemia. The p210 BCR-ABL chimeric protein is expressed only on leukemic cells and could serve as real tumor-specific antigen. We theorized that CRCL obtained from 12B1 tumor was likely to chaperone BCR-ABL derived fusion peptides. We further hypothesized that DC could cross-present BCR-ABL fusion peptides to T cells and induce specific immune responses in vivo.

MATERIALS AND METHODS

Peptides: Synthetic BCR-ABL chimeric peptide, GFKQSSKAL, and control peptide, HYLSTQSALSK, were purchased from Sigma-Genosys (Sigma-Genosys, The Woodlands, TX). The 11-mer HYLSTQSALSK peptide is processed by DC and the resulting 9-mer HYLSTQSAL is predicted to bind with high affinity to H-2K^d ⁴.

Bcr-abl-positive leukemia cell line: 12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the human *bcr-abl* (b3a2) fusion gene, and these cells express the p210 BCR-ABL protein. This is an aggressive leukemia, with the 100% lethal dose (LD₁₀₀) being 10² cells after tail vein injection and 10³ cells after subcutaneous injection. The 12B1 cell line was kindly provided by Dr. Wei Chen (Cleveland Clinic, Cleveland, OH). The cell line was tested monthly and found to be free of Mycoplasma contamination.

Mice: Six- to 10-week-old female BALB/c (H2^d) mice (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments. The animals were housed in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Tumor generation: All tissue/cell culture reagents were purchased from Gibco/BRL (Gaithersburg, MD). 12B1 cells were cultured at 37 °C and in 5% CO₂ in RPMI medium containing 10% heat-inactivated fetal calf serum and supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 0.025 µg/ml amphotericin B, 0.5 × minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, and 50 µM 2-mercaptoethanol. Cells were prepared for injection by washing and resuspending in Hanks' balanced salt solution. The cells were counted and adjusted to a concentration of 25 × 10⁶ cells/ml. Female BALB/c mice were injected with 0.2 ml (5 × 10⁶ cells) subcutaneously in both flanks and were monitored for tumor development. Tumors greater than 1 cm in diameter were harvested from euthanized mice. *In vivo* passaging of tumors involved harvesting and mincing the tumor to produce a cell suspension. The cell suspension was filtered through a Nitex screen to remove debris and centrifuged. The cell pellet was resuspended, washed, counted, and injected into mice.

FS-IEF for chaperone rich cell lysates (CRCL) enrichment : Tumor tissue grown *in vivo* was harvested from mice and homogenized at 4 °C in a motor-driven glass/Teflon homogenizer; the buffer was 10 mM Tris/Cl (pH 7.4)/ 10 mM NaCl/ 0.1% Triton X-100/0.1% Triton X-114/ 0.1% Igepal CA-630 (equivalent to Nonidet P-40; all detergents were from Sigma Chemical, St Louis, MO), with the following protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN): leupeptin (2 µg/ml), pepstatin A (1 µg/ml), phenylmethylsulfonyl fluoride (0.5 mM) and a Complete protease inhibitor cocktail tablet. This buffer was chosen for its low ionic strength and ability to solubilize membranes. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C to obtain a "low-speed" supernatant. That supernatant was centrifuged at 100,000 × g for 60 min at 4°C to obtain a "high speed" supernatant. This was then dialyzed against 5 mM Tris/Cl (pH 7.4)/ 5 mM NaCl, 0.05% Triton X-100/0.05% Triton X-114/0.05% Igepal CA-630. Protein concentration of this dialysate was determined by BCA (bicinchoninic acid) method (Pierce Endogen, Rockford, IL) using bovine serum albumin as a standard. This dialysate was frozen in aliquots containing 25 mg of total protein. To generate vaccine one aliquot was filtered through a 0.8 µm filter and prepared for isoelectric focusing by adding urea to 6 M, the detergents Triton X-100, Triton X-114, and Igepal each to 0.05%, and a mixture of Rotolytes (Bio Rad Laboratories, Hercules, CA) 5 ml each solution A and B for each pH range: pH 3.9-5.6;

4.5-6.1; and 5.1-6.8) to a total volume of around 40-50 ml. FS-IEF was carried out in a Rotofor device (Bio Rad Laboratories). Isoelectric focusing was conducted for 5 h at 15 W constant power while the apparatus was cooled with recirculating water at 4 °C; the anode compartment contained 0.1 M H₃PO₄, while the cathode compartment contained 0.1 M NaOH. Twenty fractions were harvested; the pH of each fraction was determined with a standard pH meter, and the protein content was analyzed by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described⁵. SDS-PAGE and Western blot results indicated that following FS-IEF, several fractions ranging from pH 5.1 to pH 6.0 contained HSP70, HSP90, GRP94/gp96, and CRT within them. Fractions selected to be pooled for vaccines were those that contained all four of the above HSPs. Endotoxin level of the CRCL is lower than 0.01 EU/μg of CRCL as examined by Limulus Amebocyte Lysate (LAL) assay (QCL-1000, BioWhittaker, Walkersville, MD).

Preparation of chaperone-enriched vaccines: Fractions from FS-IEF that contained substantial amounts of four chaperone proteins (HSP70, HSP90, GRP94/gp96, and calreticulin), as determined by SDS-PAGE and Western blotting, were pooled and dialyzed stepwise out of urea and detergents (starting in 0.1 × phosphate-buffered saline (PBS), 4 M urea, and 0.025% detergents, ending with 0.1 × PBS). Pooled fractions were then concentrated using Centricon devices and reconstituted in PBS. Vaccines were then passed onto an Extracti-gel D column (Pierce Endogen) to remove detergent. Protein concentrations were determined by the BCA method and the concentrated proteins were diluted to appropriate concentration for *in vivo* and *in vitro* experiments.

Generation of bone marrow-derived DCs: BALB/c mouse bone marrow DCs were generated using a slightly modified protocol from that described previously⁶. Bone marrow was harvested from femurs and tibiae and filtered through a Falcon 100 μm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in complete RPMI medium (therapeutic grade; Gibco BRL, Gaithersburg, MD), which contains 10% fetal calf serum, L-glutamine, human serum albumin, 50 μg/ml streptomycin sulfate, and 10 μg/ml gentamicin sulfate. Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/ml; Peprotech, Rocky Hill, NJ) and interleukin (IL)-4 (10 ng/ml) were added to the culture. After 6 days, the nonadherent and loosely adherent cells were harvested, washed and used for *in vivo* and *in vitro* experiments. Less than 10% of these cells were contaminated by macrophages (CD14⁺ cells).

ELISPOT Assays: Enzyme-linked immunospot (ELISPOT) assays were performed to assess the interferon γ (IFN-γ) production of splenocytes from vaccinated mice following *in vitro* stimulation with 12B1 CRCL or peptides. 10⁶ splenocytes were cultured with 50 μg/ml 12B1 CRCL or 5 μg/ml peptides in the presence of 10 ng/ml GM-CSF and IL-4 for 48 hours on Millipore MultiScreen-HA 96 well plates (MAHA S45, Millipore, Bedford, MA). The plates had been previously coated overnight with anti-IFN-γ capture antibody (10 μg/mL, clone R4-6A2, rat mAb antimouse IFN-γ; BD Pharmingen, San Diego, CA). Cells were then washed out with copious amounts of PBST (PBS + 0.05% Tween 20). Biotinylated anti-IFN-γ antibody (2 μg/mL, clone XMG1.2, rat mAb antimouse IFN-γ; BD Pharmingen) was added for two hours. Free antibody was washed out, and the plates were incubated with horseradish peroxidase (HRP)-linked avidin (ABC Elite reagent, 1 drop each of Reagent A and Reagent B per 10 ml PBS, Vector Laboratories, Burlingame, CA) for 1 hour, following extensive washing with PBST, and then washing with PBS. Spots were visualized by the addition of the HRP substrate 3-amino-9-ethylcarbazole (AEC, Sigma Chemical) prepared in acetate buffer (pH 5.0) with 0.015% hydrogen peroxide. Spots were examined using a dissecting microscope. Wells of interest

were photographed with a microscope-mounted Cool SNAP CCD camera (RS Photometrics, Tucson AZ), and images captured with RS Image, Version 1.07 (Roper Scientific, Tucson, AZ). The image of each well was electronically optimized to visualize the maximum number of spots.

Cytotoxicity assay: BALB/c mice were immunized as indicated above. Seven days after the second immunization, splenocytes from the immunized mice were harvested. The in vivo-primed splenocytes were cultured in complete RPMI for 6 days in the presence of 10 μ g/ml 12B1 CRCL and 20 U/ml IL-2. Viable cells were then collected by Ficol density centrifugation and used as effector cells. Day 5 bone marrow derived DCs were incubated with 5 μ g/ml HYLSTQSALSK or GFKQSSKAL for 3 hours; alternatively, DCs were co-cultured with 50 μ g/ml 12B1 tumor derived CRCL overnight. Cells were collected and used as target cells. Stimulated effector cells were incubated with 2×10^4 target cells at indicated ratios for 6 hours and cytolytic activity was measured by non-radioactive cytotoxicity assay (Promega, Madison, WI) following the instructions provided. The percentage of cytotoxicity was determined according to the formula provided in the kit instructions.

Immunoblotting: The absence of p210 BCR-ABL protein in the 12B1 CRCL was confirmed by Western blotting and compared to the 12B1 tumor lysates and tumor lysates from BCR-ABL⁺ murine B cell lymphoma, A20. Tumor lysates refer to the starting material of FS-IEF which were prepared as described above. An equivalent amount of protein was loaded onto the gel. Following SDS-PAGE, the gels were electroblotted to nitrocellulose using an Idea Scientific electroblotter (Minneapolis, MN). Gels were transferred in 25 mM Tris, 200 mM glycine, 20% methanol overnight at 60 V, stained with Ponceau Red and destained in TBST (50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.4). Blots were blocked in 3% non-fat dried milk in TBST for 20-60 min, followed by 3 x 5 min rinses in TBST. The protein of interest was identified using the monoclonal antibody to bcr (AB-2) clone #7C6 (Oncogene Research Products, Cambridge, MA). Primary antibody solutions were prepared in blocking solution, and blots were incubated in primary antibody for 1 hr at room temperature or 12 hr at 4° C, followed by 3 x 5 min rinses in TBST. Peroxidase conjugated goat anti mouse secondary antibody was applied for 1 hr at room temperature or 12 hr at 4° C followed by chemiluminescent detection (Super Signal, Pierce, Rockford, IL).

In vivo tumor growth experiments: Mice were injected with 10^3 viable 12B1 cells at right groin on day 0. DCs were pulsed with 100 μ g/ml HYLSTQSALSK or GFKQSSKAL in the presence of 5 μ g/ml β_2 -microglobulin (Accurate Chemical and Scientific Corporation, Westbury, NY) for 3 hours, or pulsed with 50 μ g/ml 12B1 tumor derived CRCL overnight. DCs were then harvested, washed with PBS three times, and resuspended in PBS followed by s.c. injection into mice. On day 2, a total of 5×10^5 DCs were injected per mouse subcutaneously into the left groin. As control, mice were injected with equal number of non-pulsed DCs. To exclude potential CRCL toxicity on DCs, day 6 DCs were plated in 96-well flat-bottom plates (50,000 cells/well) in the presence of increasing concentrations of 12B1 tumor derived HSP70 or CRCL (0-100 μ g/ml) for 24 hours. MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], stock solution 5 mg/ml, Sigma) at 10 μ l per well as added for an additional 4 hours. The supernatant was aspirated and the formazan crystals were solubilized in dimethylsulfoxide, followed by determination of optical densities at 560 nm and 690 nm using a microtiter plate reader. Tumor size was measured every other day with calipers once the tumors became palpable. Tumor volume was calculated using the formula: length \times width² \times $\pi/6$. Differences in mean tumor volume between groups were compared using an unpaired *t* test. In other experiments the Kaplan-Meier product-limit method was used to plot the time to mice death and the log-rank

statistic to test differences between groups ^{7,8}. Mice with tumor were euthanized at the end point listed.

RESULTS

Immunization of mice with 12B1-derived CRCL loaded DCs induces BCR-ABL specific IFN- γ secretion. To immunologically identify the presence of BCR-ABL peptides in 12B1 CRCL, IFN- γ secretion of splenocytes from immunized mice was measured by ELISPOT. Mice were immunized with DCs pulsed with either 12B1-derived CRCL, or with a BCR-ABL peptide, GFKQSSKAL, on days -14 and -7. We found that in vitro re-stimulation of splenocytes with BCR-ABL peptide from mice primed with 12B1-derived CRCL resulted in substantial IFN- γ release, although at a lower level when compared to re-stimulation with CRCL (Figure 1). These data indicate that the BCR-ABL peptides are immunologically prominent components of CRCL, and contribute to the immunogenicity of 12B1-derived CRCL. Moreover, the superior IFN- γ secretion following CRCL re-stimulation suggested that other components in CRCL may contribute to the immunogenicity of CRCL. Similarly, when splenocytes from mice immunized with BCR-ABL peptide loaded DCs were harvested and re-stimulated with either specific BCR-ABL peptide or with CRCL, there was substantial IFN- γ release, with higher levels of IFN- γ secretion by re-stimulation with the BCR-ABL peptide. Splenocytes from none of the groups secreted IFN- γ when re-stimulated with an irrelevant peptide, HYLSTQSALSK, confirming the specificity of CRCL immunization (Figure 1).

Immunization of mice with 12B1-derived CRCL loaded DCs induces BCR-ABL specific CTL activity. We further examined whether immunization of CRCL loaded DCs induced BCR-ABL specific CTL responses. Mice were immunized as described above. In vivo primed splenocytes were re-stimulated in vitro with CRCL for 6 days. Viable cells were then collected by density centrifugation and used as effector cells. BCR-ABL peptide or CRCL loaded DCs served as target cells. For controls, irrelevant peptide was loaded onto DCs. Primed splenocytes from mice immunized with either DCs loaded with BCR-ABL peptide or with CRCL displayed significant cytolytic activities against DCs pulsed with either BCR-ABL peptide or with CRCL (Figure 2). These results again indicate the presence of BCR-ABL peptides in CRCL and further suggested that BCR-ABL peptides are cross-presented to T cells by DCs. Splenocytes from mice vaccinated with either saline or empty DC displayed no cytotoxicity against any of the target cells. In addition, no significant killing was observed against irrelevant peptide pulsed DCs (Figure 2), demonstrating the specificity of CRCL or BCR-ABL peptide immunization.

Absence of BCR-ABL p210 protein in 12B1 CRCL: To exclude the possibility that BCR-ABL specific IFN- γ secretion or CTL activity of splenocytes from vaccinated mice was due to the presence of BCR-ABL protein in 12B1-derived CRCL preparation, Western Blotting was conducted. An antibody against c-ABL which recognizes the c-terminal of ABL protein but not BCR-ABL fusion peptides was used. BCR-ABL protein was absent in 12B1 CRCL (Figure 3), but present in the 12B1 lysates which were the starting material of FS-IEF. This confirms that the BCR-ABL specificity of 12B1-derived CRCL immunization was not an artificial effect of BCR-ABL protein in CRCL.

Immunization of mice with 12B1-derived CRCL loaded DCs provides superior therapeutic effects. BCR-ABL peptide based vaccine has been explored in a variety of clinical studies. Given the ability of immunization with DC pulsed with CRCL to induce BCR-ABL specific IFN- γ secretion and CTL responses, in vivo experiments were then conducted to compare the efficacy of CRCL loaded DC with that of BCR-ABL peptide loaded DCs. Mice were inoculated

subcutaneously with lethal dose of 12B1 cells (3×10^3) on day 0. Bone marrow derived DCs were incubated with 50 $\mu\text{g/ml}$ of 12B1-derived CRCL for 24 hours or with 100 $\mu\text{g/ml}$ of GFKQSSKAL or HYLSTQSALSK peptide for 3 hours. The DCs were washed and resuspended in PBS and mice were immunized subcutaneously with 5×10^5 DCs per mouse on day 2. Immunization with CRCL loaded DCs significantly inhibited tumor development in mice when compared to all other groups (Figure 4A). This therapeutic vaccine resulted in eradication of tumor growth in 60% of mice with the disease (Figure 4B). DC pulsed with BCR-ABL peptide were less effective albeit also significantly better than saline or DC pulsed with irrelevant peptide, with about 20% mice remaining tumor free (Figure 4B). These findings suggest that 12B1 tumor derived CRCL may offer an advantage as a source of tumor antigens for pulsing DCs when compared to single BCR-ABL peptide.

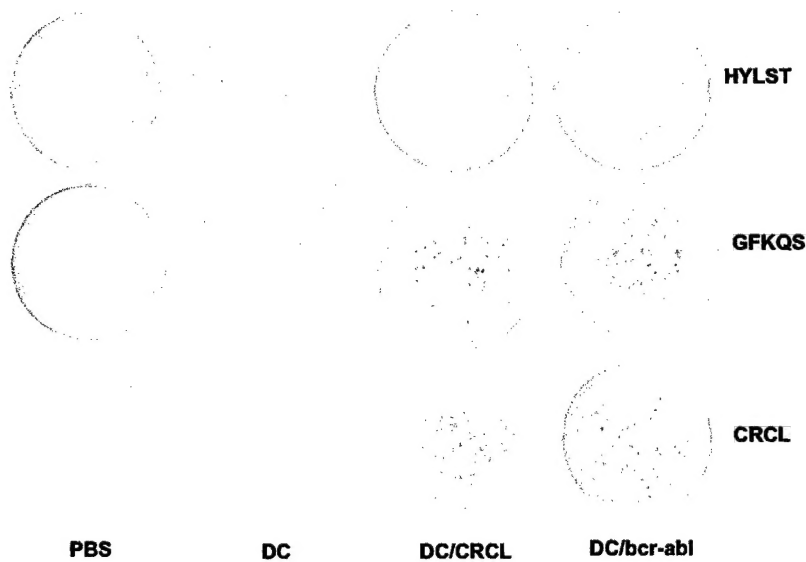


Figure 1: Immunization of mice with 12B1-derived CRCL loaded DCs induces BCR-ABL specific IFN- γ secretion. Mice were immunized with DCs (5×10^5) loaded with 12B1-derived CRCL, or with a BCR-ABL peptide, GFKQSSKAL, or with an irrelevant peptide, HYLSTQSALSK, on days -14 and -7. For controls, mice were immunized with equal volume of PBS or equal number of empty DCs. On day 0, splenocytes were collected, and re-stimulated with indicated peptide or CRCL for 48 hours. IFN- γ production was determined by ELISPOT assay.

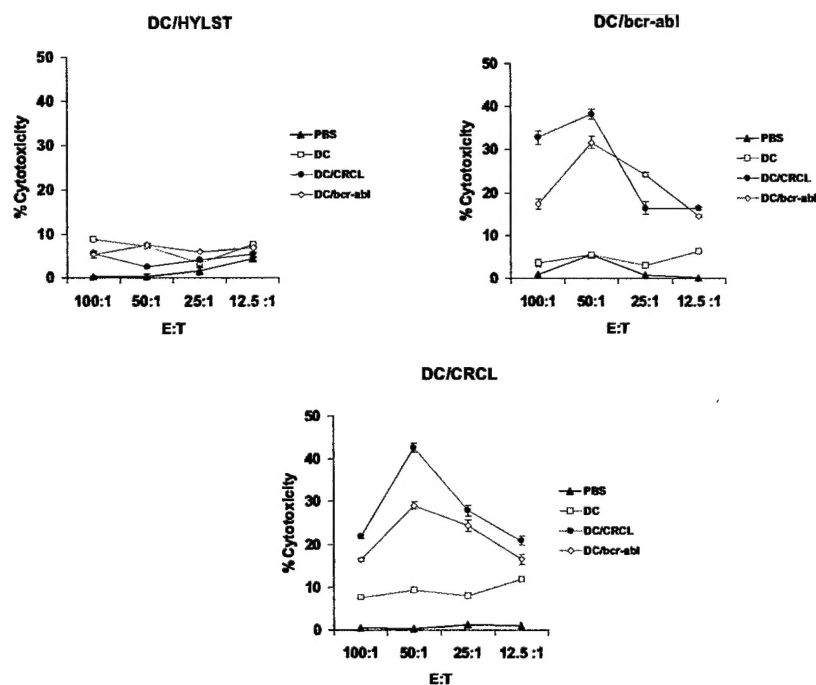


Figure 2: Immunization of mice with 12B1-derived CRCL loaded DCs induces BCR-ABL specific CTL activity. Mice were immunized as described in Materials and Methods. On day0, splenocytes were harvested and cultured in the presence of 10 μ g/ml 12B1 CRCL and 20 U/ml of IL-2 for 6 days. Viable cells were then harvested by Ficol density centrifugation and used as effector cells. Day 5 bone marrow derived DCs were incubated with 5 μ g/ml of HYLSTQSALSK or GFKQSSKAL peptide for 3 hours; alternatively, DCs were co-cultured with 50 μ g/ml 12B1 tumor derived CRCL overnight. Cells were then collected and used as target cells. Effector cells were tested for cytolytic activity against indicated target cells by non-radioactive cytotoxicity assay kit (representative data from 2 experiments is shown).

210 KD

A20 CRCL 12B1 lysates 12B1 CRCL

Figure 3: Absence of BCR-ABL p210 protein in 12B1 CRCL. 12B1 tumor lysates and 12B1 CRCL were prepared and separated on SDS-PAGE, followed by transfer to nitrocellulose for Western Blotting. Presence of p210 BCR-ABL protein was probed using an antibody for c-ABL. A20 tumor lysates was served as a negative control.

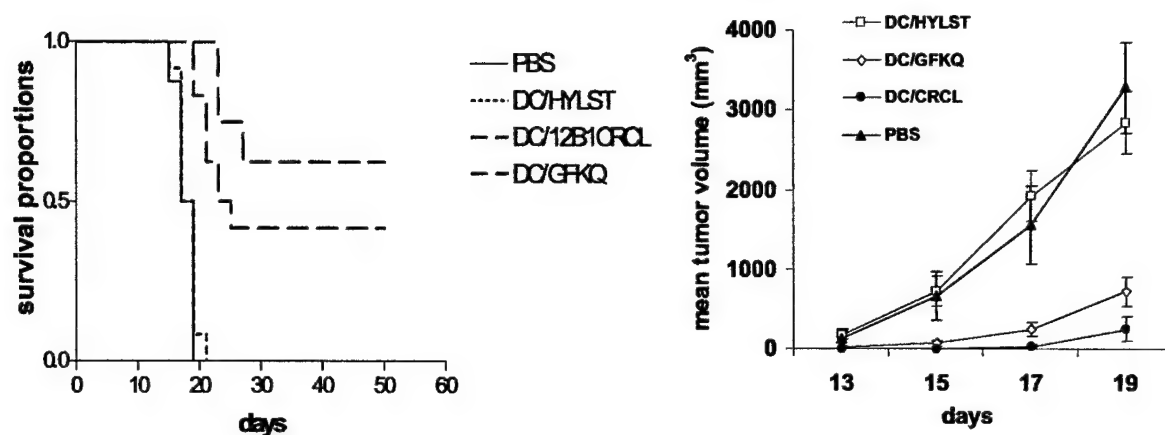


Figure 4: Immunization of mice with 12B1-derived CRCL loaded DCs provides superior therapeutic effects. (A) BALB/c mice were injected with 3×10^3 12B1 cells subcutaneously in the right groin on day 0. On day 2, mice were immunized with 5×10^5 DCs that had been loaded with 12B1-derived CRCL or indicated peptides as mentioned in Materials and Methods. Tumor volume was measured and tumor growth curves as mean tumor volume of each group are shown ($n=8-16$ mice per group, pooled data from 2 experiments are shown. PBS vs DC/CRCL, $p < 0.05$; PBS vs DC/BCR-ABL, $p < 0.05$; DC/HYLST vs DC/CRCL, $p < 0.05$; DC/HYLST vs DC/BCR-ABL, $p < 0.05$; DC/CRCL vs DC/BCR-ABL, $p < 0.05$). (B) Survival of mice was monitored and displayed in the Kaplan-Meier plot ($n=8-16$ mice per group, pooled data from 2 experiments are shown. PBS vs DC/CRCL, $p < 0.05$; PBS vs DC/BCR-ABL, $p < 0.05$; DC/HYLST vs DC/CRCL, $p < 0.05$; DC/HYLST vs DC/BCR-ABL, $p < 0.05$; DC/CRCL vs DC/BCR-ABL, $p < 0.05$).

DISCUSSION

We have previously demonstrated that immunization of mice with DCs loaded with CRCL elicits potent tumor specific immune responses in different tumor models ^{1,2}. Since chaperone proteins have been demonstrated to bind endogenous peptides ⁹⁻¹¹, we reasoned that BCR-ABL derived fusion peptides are likely to be components of the antigenic repertoire of 12B1 tumor derived CRCL. To address this hypothesis, we tested whether immunization of mice with DCs loaded with CRCL induced BCR-ABL specific immune responses in vivo. We found that splenocytes from mice immunized with DCs loaded with CRCL secreted large amounts of IFN- γ when re-stimulated with a BCR-ABL peptide, GFKQSSKAL, indicating that BCR-ABL peptides, either this specific 9 mer peptide, GFKQSSKAL, and / or its longer precursors, are chaperoned by 12B1 tumor derived CRCL. Very interestingly, we found that these splenocytes secreted higher amounts of IFN- γ upon re-stimulation with 12B1 derived CRCL than that with BCR-ABL peptide. These findings were further confirmed when splenocytes from mice immunized with BCR-ABL peptide loaded DCs secreted higher levels of IFN- γ when re-stimulated with 12B1-derived CRCL than that with BCR-ABL peptide. One reasonable explanation to the superior IFN- γ production could be that other antigenic peptides are also chaperoned by CRCL. The potential candidate antigens chaperoned by CRCL may include Wilms tumor antigen ¹², minor histocompatibility antigen ^{13,14}, protease 3 ¹⁵, which have been documented to be potential CML tumor associated antigens. Experiments are ongoing in our lab to identify the antigenic components of CRCL chaperoned peptide repertoire. In addition to the broader antigenic spectrum, the adjuvant effects of CRCL ^{2, 16, 17} may also contribute to the superior IFN- γ production following vaccination with DCs pulsed with CRCL.

DCs are professional APCs, known to be critical activators of T cell responses. Although the mechanisms are not completely clear, an increasing body of data suggests that DCs take up chaperone-peptide complexes through specific receptors and re-present the peptides on MHC class I molecules¹⁸⁻²². Following uptake, the chaperone protein-escorted peptides are processed and re-presented on MHC class I molecules to generate antigen-specific CTLs. BCR-ABL peptides, which are chaperoned by 12B1 CRCL as indicated above, have been previously reported to be able to bind MHC-I and MHC-II molecules following uptake by DCs. We therefore hypothesized that BCR-ABL peptides are cross-presented to MHC class I molecules for T cell priming. We found that splenocytes from mice immunized with CRCL loaded DCs specifically lysed BCR-ABL peptide coated DCs, but not irrelevant peptide coated DCs. Similarly, splenocytes from mice immunized with BCR-ABL peptide loaded DCs lysed CRCL coated DCs (Figure 2). These findings clearly demonstrate that immunization of 12B1-derived CRCL induce BCR-ABL specific CTLs. They further indicate that DCs process CRCL chaperoned antigens and cross-present the escorted antigenic peptides, including the BCR-ABL peptides to T cells in vivo.

Together, we have immunologically identified that BCR-ABL peptides are present in 12B1-derived CRCL and are cross-presented by DCs to generate BCR-ABL specific CTLs in vivo. The absence of BCR-ABL protein in CRCL as demonstrated by Western Blotting confirmed that the BCR-ABL specific immune responses induced by CRCL immunization were not due to adventitious vaccination with the p210 protein.

The efficacy of BCR-ABL peptide based immunotherapy has been widely studied^{23,24}. We have previously shown that immunization of mice with DCs loaded with the synthetic BCR-ABL chimeric nonapeptide, GFKQSSKAL, generates BCR-ABL -specific CTL in vivo²⁵. *Bcr-abl* fusion gene is the primary mutation that leads to malignant transformation; however, secondary mutations often occur²⁶. Therefore, leukemic cells can easily escape immune responses generated by single peptide vaccination. On the contrary, since CRCL may carry multiple antigenic peptides, therefore immunization of CRCL may induce more potent immunity when compared to single peptide immunization. In addition, CRCL have been shown to activate DCs², and to enhance the immunogenicity of apoptotic tumor cells¹⁶. With these adjuvant effects, CRCL are likely to confer superior immunogenicity compared to single peptide. We tested this by comparing the potency of immunization with DCs loaded with BCR-ABL peptide or with 12B1-derived CRCL. We showed that vaccination with DCs loaded with CRCL led to significantly higher percentage of mice survival.

Task 2. STUDY THE IN VIVO SYNERGISTIC EFFECTS OF CRCL VACCINE/ADJUVANT WITH STI-571, A TYROSINE KINASE INHIBITOR THAT INDUCES APOPTOSIS IN MURINE 12B1 BCR-ABL⁺ CELLS.

The remarkable record of imatinib mesylate against CML in chronic phase is tempered by its reduced effectiveness against the disease in the accelerated phase or during blastic transformation^{27, 28, 29}, or by the increasing number of cases where drug resistance develops^{30, 31}. These situations have led to drug combination approaches to augment the activity of imatinib via alternative targeting of either the p210 proteins or other important downstream signal transduction molecules^{32, 33, 34, 35, 36}. However, there have been few attempts to combine imatinib with immunotherapy³⁷, and there are no published reports on utilization of vaccine therapy in conjunction with imatinib either in human trials or in animal models. On the other hand, imatinib mesylate has been reported to be able to enhance the antigen-presenting

capacities of DCs, suggesting that imatinib may be used in the immunotherapy of cancer³⁸. Our previous reports have shown that multiple chaperone proteins may be enriched into a vaccine form from tumor cell lysates by a free-solution isoelectric focusing (FS-IEF) method³⁹. We refer to these vaccines as chaperone-rich cell lysates (CRCL), and have reported that they are potent immunologic agents against a variety of murine tumors^{39,1}, including the 12B1 BCR-ABL⁺ leukemia^{1,2}. In this current work we show that the combination of imatinib with cellular vaccines of dendritic cells pulsed with 12B1-derived CRCL yields anti-12B1 specific T cells, and potent therapeutic anti-tumor activity resulting in tumor-free survival in a high percentage of mice. This report suggests that CRCL vaccine may be effectively combined with imatinib mesylate to treat *bcr-abl*⁺ leukemia.

MATERIALS AND METHODS

Bcr-abl-positive leukemia cell line: 12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the human *bcr-abl* (b3a2) fusion gene. This is an aggressive leukemia, with the 100% lethal dose (LD₁₀₀) being 10² cells after tail vein injection (i.v.) and 10³ cells after subcutaneous injection (s.c.)^{1,25}. The 12B1 cell line was kindly provided by Dr. Wei Chen (Cleveland Clinic, Cleveland, OH). The cell line was tested monthly and found to be free of Mycoplasma contamination. A20 cells are leukemia/lymphoma cells syngeneic to BALB/c mice⁴⁰.

Mice: Six- to 10-week-old female BALB/c (H2^d) mice (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments. The animals were housed in micro-isolation in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Preparation of imatinib mesylate for murine use: Commercially available imatinib capsule contents (Gleevec/GlivecTM, Novartis Pharmaceutical AG, Basel, Switzerland) were dissolved in distilled water (Sigma Chemical, St Louis, MO) at desired concentrations, aliquoted and stored at -20°C for future in vitro and in vivo experiments.

Imatinib mesylate treatment of in vitro grown cells: Tissue cultured 12B1 or A20 cells were plated in 96-well flat-bottom plates (50 000/well) in the presence of increasing concentrations of imatinib for 24 hours. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma), stock solution 5 mg/mL, at 10 µL per well was added for an additional 4 hours. The supernatant was aspirated and the formazan crystals were solubilized in dimethylsulfoxide, followed by determination of optical densities at 560 nm and 690 nm using a microtiter plate reader (BioMetallics, Princeton, NJ).

Terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) assay: Apoptosis induction of 12B1 cells *in vivo* was determined by TUNEL assays using an APO-DIRECTTM kit (BD PharMingen, San Diego CA). BALB/c mice were s.c. injected with 3×10³ viable 12B1 cells in the right groin on day 0. When tumor diameters reached around 2 to 3 mm, mice were treated with phosphate-buffered saline (PBS) or single dose of 900 mg/kg imatinib by gavage. Tumors were removed 24 hours later, embedded in O.C.T (Sakura Finetek U.S.A., Torrance, CA), frozen at -80 °C, cut to 5-µm-thick sections and then mounted on microscope slides and stored at -80 °C. Frozen slides were fixed in freshly prepared 1% paraformaldehyde (Sigma) in PBS for 15 minutes on ice. They were then rinsed with PBS once, and submerged in 70% cold ethanol for 30 minutes on ice. Following two rinses in PBS, slides were allowed to dry. TUNEL

staining solution was added to the slides and they were incubated in a humidified chamber at 37 °C for 60 minutes followed by washing with rinsing buffer for 3 times. Ribonuclease/propidium iodide solution was then added to the slides and they were incubated in the dark for 10 minutes. After 2 washes with PBS, they were mounted using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and covered with cover slips. Samples were examined using a Bio-Rad 1024 MRC confocal imaging system (Bio-Rad Laboratories, Hercules, CA).

Annexin V staining of Imatinib mesylate-treated cells: 12B1 cells in culture were treated with 2 μ M of imatinib for 6 or 20 hours. Cells were then collected and stained with Annexin V-Fluoroisothiocyanate (FITC) and propidium iodide (PI) using the Annexin-V-FLUOS staining kit (Roche Molecular Biochemicals, Indianapolis, IN) followed by flow cytometric analysis of cells (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Western blotting of 12B1 cell lysates for BCR-ABL protein and phosphotyrosine content.: 12B1 cells were treated in vitro with 2 μ M of imatinib for time points from 0-24 hours. Cells were harvested by centrifugation and lysed in TNES protein lysis buffer (0.05 M Tris, 1% NP-40, 2.5mM EDTA, 0.1M sodium chloride, Sigma) containing 2mM sodium orthovanadate (Sigma). Protein concentrations were determined by BCA assay (Pierce Endogen, Rockford IL), and 30 μ g lysate from each time point was loaded onto 10% sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE), electrophoresed, and electroblotted to nitrocellulose. The membranes were probed with antibodies to c-abl (2 μ g/ml, Ab-3, Oncogene Research Products, San Diego, CA) or to phosphotyrosine-containing proteins (clone 4G10, Upstate Biotechnology, Lake Placid, NY) followed by appropriate secondary antibodies. Positive control for tyrosine-phosphorylated proteins was an EGF-stimulated A431 cell lysate provided by Upstate Biotechnology. Membranes were developed with enhanced chemiluminescent substrates (Pierce Endogen).

Heat shock protein (HSP) 70 and 60 expression in imatinib mesylate- treated 12B1 cells: 12B1 cells were treated in vitro with 2 μ M of imatinib for overnight. HSP70 and 60 expression on 12B1 cell surface and the total HSP70 and 60 expression in 12B1 cells were examined by flow cytometry and western blotting respectively as described previously⁴¹, with a different purified anti-HSP70 (clone N27F3-4, mouse IgG₁; StressGen, Victoria, Canada) and a different secondary antibody: Alexa Fluor 488 F(ab')₂ fragment goat anti-mouse IgG(H+L) (Molecular Probes, Eugene, Oregon).

Imatinib mesylate treatment of mice: Mice were treated with the indicated doses of imatinib every morning and every evening from day 2 to day 9 by gavage. Imatinib was administered in a volume of 300 μ L sterile water by means of straight animal feeding needles (Popper and Sons, Inc. New Hyde Park, NY). The treatment regimens were well tolerated.

Tumor generation: All tissue/cell culture reagents were purchased from Gibco/Invitrogen (Gaithersburg, MD). 12B1 and A20 cells were cultured at 37 °C and in 5% CO₂ in RPMI medium containing 10% heat-inactivated fetal calf serum and supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, 0.025 μ g/ml amphotericin B, 0.5 \times minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, and 50 μ M 2-mercaptoethanol. Cells were prepared for injection by washing and resuspending in Hanks' balanced salt solution. The cells were counted and adjusted to a concentration of 25 \times 10⁶ cells/ml. Female BALB/c mice were injected with 0.2 ml (5 \times 10⁶ cells) subcutaneously in both flanks and were monitored for tumor development. Tumors greater than 1 cm in diameter were harvested from euthanized mice. *In vivo* passaging of tumors for biochemical preparations or for tumor challenge involved harvesting and mincing the tumor to produce a cell suspension.

The cell suspension was filtered through a Falcon 100 μ m nylon strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove debris and centrifuged. The cell pellet was resuspended, washed, counted, and injected into mice.

Free solution-isoelectric focusing (FS-IEF) for chaperone-rich cell lysates (CRCL) generation: Tumor tissue grown *in vivo* was harvested from mice and homogenized at 4 °C in a motor-driven glass/Teflon homogenizer; the buffer was 10 mM Tris-Cl (pH 7.4)/ 10 mM NaCl/ 0.1% Triton X-100/0.1% Triton X-114/ 0.1% Igepal CA-630 (equivalent to Nonidet P-40; all detergents were from Sigma), with the following protease inhibitors (Roche Molecular Biochemicals): leupeptin (2 μ g/ml), pepstatin A (1 μ g/ml), Perfabloc (0.5 mM) and a Complete protease inhibitor cocktail tablet. This buffer was chosen for its low ionic strength and ability to solubilize membranes. The homogenate was centrifuged at 10,000 x g for 30 min at 4°C to obtain a "low-speed" supernatant. That supernatant was centrifuged at 100,000 x g for 60 min at 4°C to obtain a "high speed" supernatant. This was then dialyzed against 5 mM Tris/Cl (pH 7.4)/ 5 mM NaCl, 0.05% Triton X-100/0.05% Triton X-114/0.05% Igepal CA-630. Protein concentration of this dialysate was determined by BCA method (Pierce Endogen) using bovine serum albumin as a standard. This dialysate was frozen in aliquots containing 25 mg of total protein. To generate vaccine one aliquot was filtered through a 0.8 μ m filter and prepared for isoelectric focusing by adding urea to 6 M, the detergents Triton X-100, Triton X-114, and Igepal each to 0.05%, and a mixture of pH gradient buffer pairs (5 ml of each member of each pair): pH 3.9-5.6, 200 mM MES (2-[4-morpholino]-ethane sulfonic acid and 200 mM glycyl-glycine, Fisher Scientific, Fair Lawn, NJ); pH 4.5-6.1, 200 mM MOPSO (3-[n-morpholino]-2-hydroxy-propanesulfonic acid and 200 mM α -alanine, Sigma); pH 5.1-6.8, 200 mM TAPS (2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino-1-propanesulfonic acid, Fisher) and 200 mM ϵ -amino-n-caproic acid (Sigma). Water was added to a total volume of 50-60 ml. FS-IEF was carried out in a Rotofor device (Bio Rad Laboratories). Isoelectric focusing was conducted for 5 h at 15 W constant power while the apparatus was cooled with recirculating water at 4 °C; the anode compartment contained 0.1 M H₃PO₄, while the cathode compartment contained 0.1 M NaOH. Twenty fractions were harvested; the pH of each fraction was determined with a standard pH meter, and the protein content was analyzed by SDS-PAGE and Western blotting as previously described³. SDS-PAGE and Western blot results indicated that following FS-IEF, several fractions ranging from pH 5.1 to pH 6.0 contained HSP70, HSP90, GRP94/gp96, and calreticulin within them^{42, 2}. Fractions selected to be pooled for vaccines were those that contained all four of the above HSPs. FS-IEF utilizes small amounts of starting material to yield relatively large amounts of tumor-derived chaperone proteins. In general, 1g of tumor can yield 1000 μ g CRCL vaccine while from the same amount of tumor, only 30-50 μ g of individual chaperone protein such as HSP70 can be generated using conventional purification strategies. Endotoxin level of the CRCL is lower than 0.01 endotoxin units/ μ g of CRCL as examined by Limulus Amebocyte Lysate assay (QCL-1000, BioWhittaker, Walkersville, MD).

Fractions from FS-IEF that contained substantial amounts of four chaperone proteins (HSP70, HSP90, GRP94/gp96, and calreticulin), as determined by SDS-PAGE and Western blotting, were pooled and dialyzed stepwise out of urea and detergents (starting in 0.1 X PBS, 2 M urea, and 0.025% detergents, ending with 0.1 X PBS). Pooled fractions were then concentrated using Centricon devices (Millipore, Bedford, MA) and reconstituted in PBS. Vaccines were then passed onto an Extracti-gel D column (Pierce Endogen) to remove detergent. Protein concentrations were determined by the BCA method and the concentrated proteins were diluted to appropriate concentration for *in vivo* and *in vitro* experiments.

Generation of bone marrow-derived DCs: BALB/c mouse bone marrow DCs were generated using a slightly modified protocol from that described previously⁶. Bone marrow was harvested from femurs and tibiae and filtered through a Falcon 100 μ m nylon cell strainer (Becton Dickinson Labware). Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in complete RPMI medium (therapeutic grade; Gibco/Invitrogen), which contained 10% fetal calf serum, L-glutamine, 50 μ g/ml streptomycin sulfate, and 10 μ g/ml gentamicin sulfate. Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/ml; Peprotech, Rocky Hill, NJ) and interleukin (IL)-4 (10 ng/ml) were added to the culture. After 6 days, the nonadherent and loosely adherent cells were harvested, washed and used for *in vivo* and *in vitro* experiments. Less than 10% of these cells were contaminated by macrophages (CD14⁺ cells) as determined by flow cytometry.

***In vivo* tumor growth experiments:** Mice were injected with 3×10^3 (LD₁₀₀) viable 12B1 cells in the right groin on day 0. 12B1 cells were obtained from one single *in vivo* passage. On day +2, mice began treatment with imatinib by gavage (200, 300, or 400 mg/kg) twice per day for 7 days. In experiments with DCs, day 5 DCs were incubated with 50 μ g/ml CRCL vaccines in the presence of 10 ng/ml murine GM-CSF and 10 ng/ml murine IL-4 for 24 hours, then the DCs were washed with PBS three times, and resuspended in PBS followed by s.c. injection into the left groin of mice. A total of 5×10^5 DCs were injected per mouse. Tumor size was measured every other day with calipers once the tumors became palpable. Tumor volume was calculated using the formula: (length) \times (width)² \times ($\pi/6$). Differences in mean tumor volume between groups were evaluated using an unpaired *t* test.

Interferon-gamma (IFN- γ) secretion, interleukin 2 (IL-2) production and mixed lymphocyte reaction (MLR) of splenocytes from treated mice: BALB/c mice were injected with 3×10^3 viable 12B1 cells on day 0. Mice were then treated as indicated above. On day 12, splenocytes were harvested. IFN- γ and IL-2 production and MLR of splenocytes were examined as described previously².

Cytotoxicity assay BALB/c mice were treated as indicated above. On day 14, splenocytes were harvested and cultured in RPMI complete media in the presence of 10 μ g/ml CRCL and 20 U/ml IL-2 for 7 days. Viable cells were then harvested by Ficoll density centrifugation (Sigma) and used as effector cells. Stimulated effector cells were tested for cytolytic activity against 12B1, or A20 cells using a Cytotox 96[®] Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) following the instructions provided. The percentage of cytotoxicity was determined according to the formula provided in the kit instructions by the manufacturer. One lytic unit (LU) was defined as the number of effectors required to lyse 40% of targets; cytotoxicity is presented as LU per 10^6 effector cells.

RESULTS

12B1 cells show *in vitro* and *in vivo* sensitivity to imatinib mesylate. Since 12B1 cells are murine bone marrow-derived cells transduced with the human bcr-abl cDNA encoding the (b₃a₂) p210 BCR-ABL protein, we anticipated that these cells would be sensitive to imatinib exposure. 12B1 cells grown for 24 hours in increasing concentrations of imatinib showed a dose-dependent reduction in viability as measured by MTT assay (Figure 1 A). A20 cells, a syngeneic murine

leukemia/lymphoma (i.e., lacking the *bcr-abl* transgene), were insensitive to the drug at all concentrations tested.

We further demonstrated that imatinib induced apoptosis of 12B1 cells both *in vitro* and *in vivo*. 12B1 cells exposed to 2 μ M imatinib stained with prominent amounts of Annexin V on their cell surface with increasing exposure to the drug (Figure 1 B). Flow cytometric analysis indicated that after 6 hours incubation, about 30% of 12B1 cells were Annexin V positive, which increased to over 55% by 20 hours, while half of these cells were moving into secondary necrosis (PI and Annexin positive). Comparable proportions of 12B1 cells underwent apoptosis when treated with 4 μ M imatinib (data not shown). Apoptotic induction of 12B1 cells *in vivo* was further confirmed by TUNEL staining of tumors harvested from mice receiving imatinib treatment (Figure 1 C).

Imatinib mesylate inhibits 12B1 bcr-abl tyrosine kinase phosphorylation in vitro. To examine whether the 12B1 susceptibility to imatinib may lie in the phosphorylation status of p210, 12B1 cells were exposed to 2 μ M of imatinib for 0-24 hrs, and the cells were harvested at time points shown (Figure 2). Cell lysates were prepared and separated on SDS-PAGE, followed by transfer to nitrocellulose for Western blotting. We observed that the p210 BCR-ABL protein was present to an appreciable extent in cells at all of the time points chosen (as demonstrated by probing of the blot with an antibody for c-abl, Figure 2, top). However, the p210 protein displayed progressively less tyrosine phosphorylation (when probed with anti-phosphotyrosine antibodies, Figure 2, bottom). The reduced phosphorylation status of p210 may result in reduced activation of downstream signaling molecules which may override anti-apoptotic mechanisms employed by BCR-ABL positive leukemic cells.

Imatinib mesylate reduces tumor burden and prolongs survival but does not cure mice of the disease. We next examined the efficacy of imatinib treatment of mice bearing 12B1 tumors. In dose escalation studies, we found that increasing amounts of imatinib resulted in significant retardation of tumor growth (Figure 3 A). However, all animals eventually succumbed to disseminated disease or to large tumor masses (Figure 3 B). For future treatments, we chose 300 mg/kg of imatinib twice daily for 7 days, since the effects were comparable to higher drug doses, and this was already a relatively high dose for mice when compared to other reports⁴³.

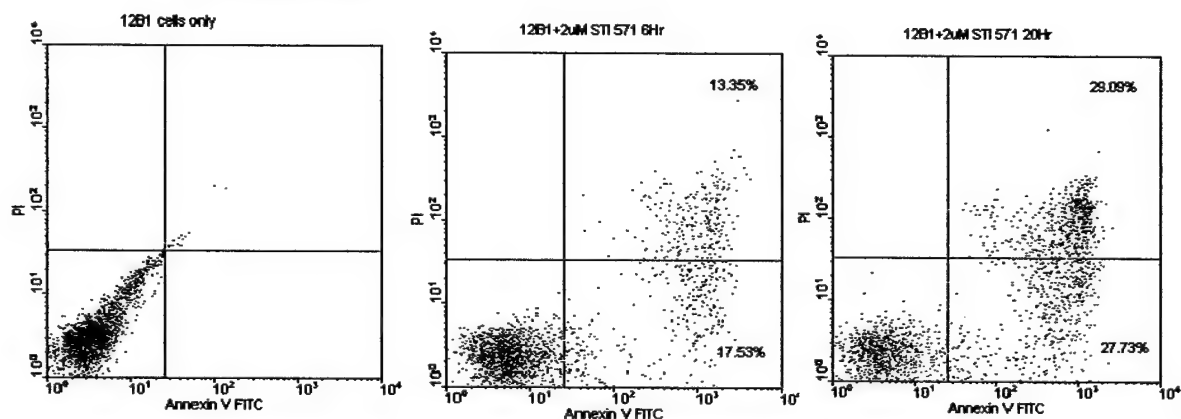
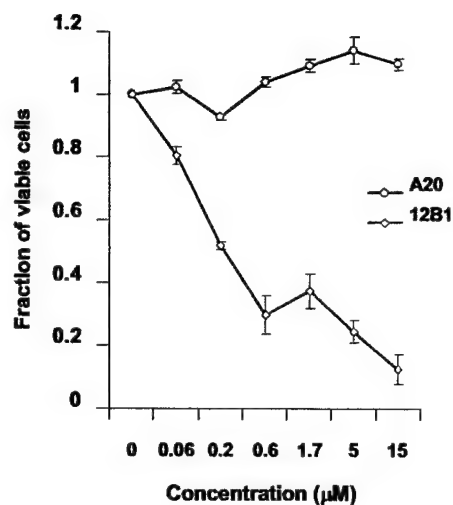
Combination therapy of imatinib mesylate with DCs pulsed with 12B1-derived CRCL can be curative against mice bearing 12B1 tumor. We wished to examine whether combination of specific chemotherapy with specific immunotherapy may offer an advantage over monotherapy. Since imatinib is not particularly myelosuppressive, and may actually enhance antigen presenting cell (APC) activity³⁸, combining it with an immunostimulatory vaccine such as CRCL pulsed DCs seemed like a feasible approach, particularly in light of the success we previously had using this vaccination approach alone^{1,2}. Mice were inoculated with tumor on day 0, and were treated with imatinib as above (i.e., 300 mg/kg gavage twice daily) from days 2-9. DCs pulsed with 12B1-derived CRCL (DC/CRCL, 5×10^5 cells/ mouse) were injected s.c. on day 2. Tumor volume was thereafter monitored. Figure 4 shows individual tumor growth curves of mice in each treatment group. All mice in the PBS control or imatinib alone groups developed tumor. However, DC/CRCL alone or combination therapy resulted in eradication of tumor growth in 3/8 and 4/8 mice, respectively. The mean tumor volume of each group was further analyzed. Each of the three treatment regimens (imatinib alone, DC/CRCL alone, or the combination) significantly delayed the mean tumor growth when compared to saline-treated controls. Moreover, treatment with imatinib + DC/CRCL was superior to imatinib or DC/CRCL alone ($p < 0.05$). These data were reproduced in an additional experiment (data not shown) with survival analysis of the pooled data demonstrating a survival of 63% of mice in the combination

therapy group, which compared favorably to 44% for the group treated with immunotherapy alone, and 6% in the imatinib group.

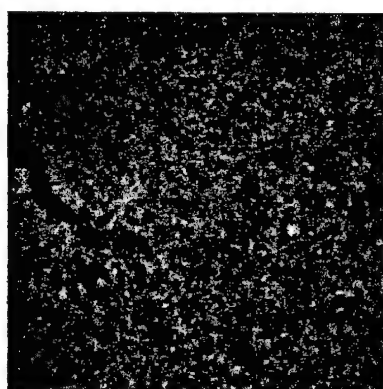
Combination therapy of imatinib and DC/CRCL induces superior IFN- γ secretion and cytotoxic T lymphocyte (CTL) activity in vivo. We have previously shown that vaccination with DC/CRCL induced T cell dependent immunity². In addition, splenocytes from vaccinated mice displayed tumor antigen specific CTL activity and secreted higher amounts of IFN- γ following *in vitro* restimulation with tumor antigen or antigenic-peptide bearing chaperones^{1,2}. In this study, we investigated the IFN- γ secretion by splenocytes and the generation of tumor-specific CTL activity following treatment of mice with imatinib and/or DC/CRCL. Mice were inoculated with tumor on day 0, and received either DC/CRCL alone, imatinib alone, or both. On day 14, spleens were harvested, and splenocytes were restimulated with 12B1-derived CRCL. IFN- γ ELISPOT assays were performed. Only splenocytes from mice receiving DC/CRCL (with or without imatinib) showed IFN- γ production following restimulation. Interestingly, there was a sharp increase in IFN- γ production when mice received the imatinib + DC/CRCL combination. Quantitatively, there was a 2-fold increase in IFN- γ -producing cells in spleens of mice receiving combination therapy versus those receiving DC/CRCL alone (Figure 5 A). Moreover, we found increased IL-2 production and T cell proliferation in splenocytes from mice receiving combination therapy when compared to those receiving imatinib or DC/CRCL alone (Zeng et al., unpublished data).

CTLs play important roles in controlling tumor growth. To measure specific CTL activity, splenocytes from treated mice were restimulated *in vitro* with 12B1 CRCL for 7 days and then tested for cytolytic function against 12B1 tumor or against syngeneic control A20 tumor targets. DC/CRCL alone and combination therapy elicited comparable CTL activity against 12B1 tumor target but not against A20 tumor cells (Figure 5 B). In contrast, imatinib alone or saline failed to generate CTL activity.

To more accurately assess the increase in cytotoxic activity taking place *in vivo*, we therefore determined the total cytotoxic activity per spleen and presented this as lytic units. Mice treated with imatinib + DC/CRCL had a 2-fold increase in lytic units per spleen when compared to mice treated with DC/CRCL alone, and yielded a 7-fold increase when compared to imatinib (Figure 5 C).



PBS



Imatinib

Figure 1. 12B1 cells are sensitive to imatinib mesylate. (A) In vitro sensitivity of 12B1 cells to increasing concentrations of imatinib. 12B1 cells or A20 cells (5×10^4) were grown in indicated concentrations of imatinib for 24 hours. Cells viability was evaluated by MTT assays. (B) Imatinib mesylate induces apoptosis of 12B1 cells *in vitro*. 12B1 cells were exposed to 2 μ M imatinib for 6 or 20 hours. Apoptosis induction was measured by Annexin V and PI staining followed by flow cytometric analyses. (C) Imatinib mesylate induces apoptosis of 12B1 cells *in vivo*. BALB/c mice were s.c. injected with 3×10^3 viable 12B1 cells on day 0. When tumor diameters reached 2 to 3 mm, mice were treated with PBS or a single dose of 900 mg/kg imatinib by gavage. Tumors were removed 24 hours later. Apoptotic induction of 12B1 cells was detected by TUNEL staining followed by examination under confocal laser microscopy. Original magnification $\times 20$. Representative data from 3 experiments are shown

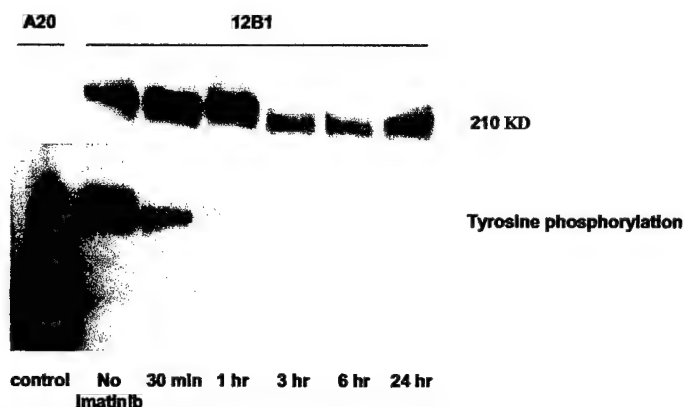


Figure 2. Imatinib mesylate inhibits 12B1 BCR-ABL tyrosine kinase phosphorylation. 12B1 cells were exposed to 2 μ M of imatinib mesylate for 0-24 hrs, and cells were harvested at the time points shown. Cell lysates were prepared and separated on SDS-PAGE, followed by transfer to nitrocellulose for Western blotting. Presence of p210 BCR-ABL protein was probed using an antibody for c-abl. A20 cell lysates was served as a negative control. The same membrane was further probed for tyrosine phosphorylation with an anti-phosphotyrosine antibody. Positive control (indicated as "control" in the lower panel) for phosphorylated tyrosine is EGF-stimulated A431 cell

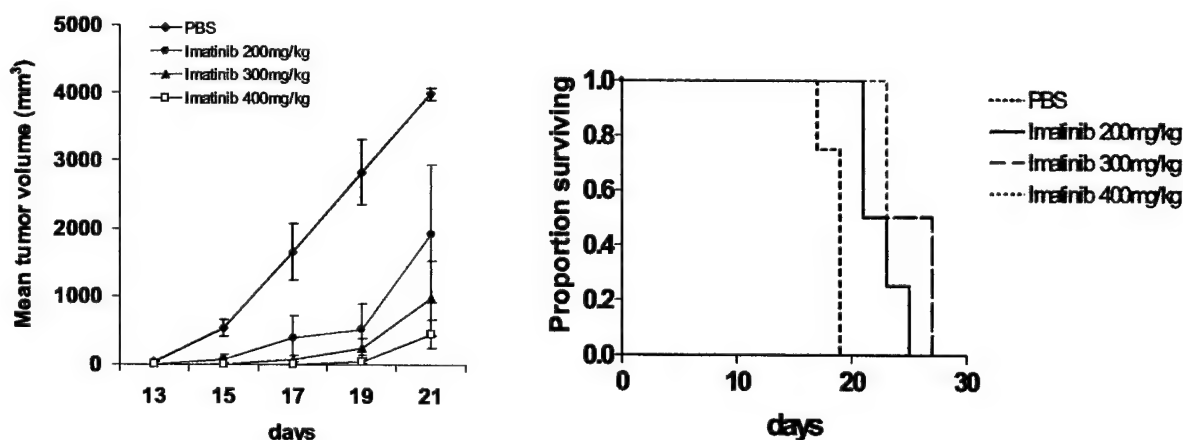


Figure 3. Treatment with imatinib mesylate reduces tumor burden and prolongs survival of mice bearing 12B1 leukemia. (A) BALB/c mice were injected with 3×10^3 12B1 cells subcutaneously in the right groin on day 0. Mice were given 200-400 mg/kg imatinib via gavage twice a day from days 2-9. Tumor volume was measured (PBS vs all imatinib treatment groups, $p < 0.05$; no significant difference between the imatinib treatment groups). (B) Survival of imatinib-treated mice was monitored (PBS vs all doses of imatinib: $p < 0.05$; no significant differences between the imatinib treatment groups).

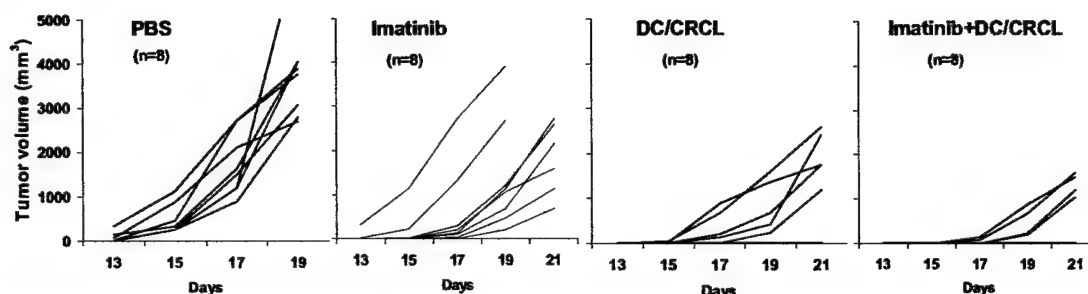


Figure 4. Combining imatinib mesylate with DCs pulsed with tumor-derived CRCL delays 12B1 tumor growth. BALB/c mice were injected with 3×10^3 12B1 cells subcutaneously in the right groin on day 0. Mice were then treated with 300 mg/kg imatinib twice daily for 7 days beginning on day 2, or with a single DC/CRCL vaccination on day 2, or with imatinib + DC/CRCL. Tumor growth curves of individual mice in each treatment group are shown (representative data from one of two experiments are shown). For mean tumor volume of each treatment: PBS vs all treatment groups: $p < 0.05$; imatinib vs DC/CRCL: $p < 0.05$; imatinib vs combination therapy: $p < 0.05$; DC/CRCL vs combination therapy: $p < 0.05$ from day 21 on).

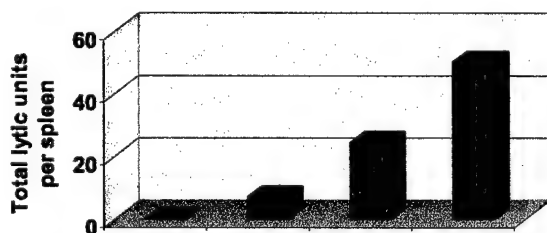
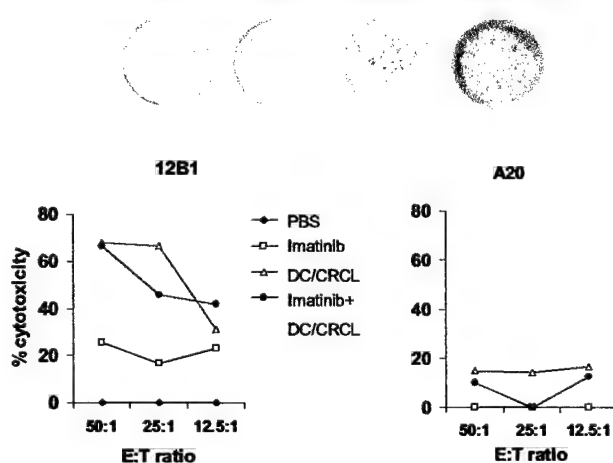
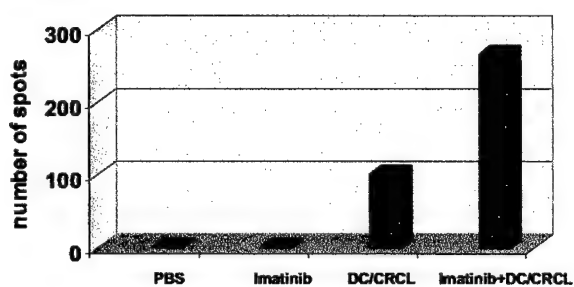


Figure 5. Imatinib mesylate and DC/CRCL combination therapy stimulate IFN- γ secretion and CTL activity. BALB/c mice were injected with 3×10^3 viable 12B1 cells on day 0. Mice were then treated with 300 mg/kg imatinib twice daily for 7 days, or with DC/CRCL on day 2, or with imatinib + DC/CRCL. Splenocytes were harvested on day 14. (A) IFN- γ secretion by splenocytes was measured using ELISPOT assays (representative data from 3 experiments are shown). PBS or imatinib vs DC/CRCL or combination therapy: $p < 0.05$; DC/CRCL vs combination therapy: $p < 0.05$. (B) Splenocytes were cultured in complete RPMI in the presence of 10 μ g/ml 12B1 CRCL and 20 U/ml IL-2 for 7 days. Viable cells were then harvested by Ficoll density centrifugation and used as effector cells. Effector cells were tested for cytolytic activity against 12B1 or A20 cells using a nonradioactive cytotoxicity assay (representative data of 2 experiments are shown). (C) Total lytic units were calculated and plotted (representative data of 2 experiments are shown; PBS vs all treatment groups: $p < 0.05$; imatinib vs DC/CRCL: $p < 0.05$; imatinib vs combination therapy: $p < 0.05$; DC/CRCL vs combination therapy: $p < 0.05$).

DISCUSSION

Combining imatinib mesylate with other effective agents appears vital since drug resistance to imatinib is clearly an emerging problem in CML^{30,31}. In addition, the ability of imatinib mesylate to enhance the function of APCs makes it an ideal drug for the combination therapy with immunotherapy³⁸. In this study we found that adding an effective vaccine approach to imatinib treatment can significantly improve survival of mice bearing 12B1 tumors. 12B1 is an aggressive murine *bcr-abl*⁺ leukemia; inoculation of mice with as few as 10² cells i.v. or 10³ cells s.c. is uniformly lethal with a median survival of 20-25 days^{1,2,25}. Combination treatment with imatinib and DCs pulsed with 12B1-derived CRCL was able to cure up to 63% of mice with 12B1 tumors. Moreover, mice receiving combination therapy were found to have higher splenic IFN- γ production and increased CTL activity when compared to those receiving imatinib or DC/CRCL alone.

Imatinib has been shown to induce apoptosis in *bcr-abl*⁺ leukemia cells^{44, 45, 46}. In our study, imatinib induced apoptosis of 12B1 cells both *in vitro* and *in vivo* as demonstrated by Annexin V staining and TUNEL assay; however, no measurable immune responses were generated by these apoptotic cells. Our data are consistent with a recent study demonstrating the failure of imatinib in inducing PR1 specific CTLs in CML patients⁴⁷. We have previously reported that apoptotic 12B1 cells are not immunogenic^{41,16}. Heat shock treatment, which leads to increased cell membrane HSP60 and inducible HSP70 expression, converted non-immunogenic apoptotic 12B1 cells into immunogenic ones. Imatinib upregulated total cellular HSP70 expression as measured by Western blotting. However, we observed only a minimal increase in membrane HSP70 as determined by flow cytometry. HSP60 levels were not affected by imatinib treatment (Zeng et al., unpublished data). These data may partly explain the lack of immune responses in imatinib treated mice. We have previously shown that CRCL derived from liver could act as a potent adjuvant if utilized in conjunction with apoptotic tumor cells¹⁶. It is therefore likely that the imatinib-induced apoptosis of 12B1 releases tumor antigens, and when given in combination with CRCL that has both antigenic as well as adjuvant effects, leads to an enhanced anti-tumor immune response.

The use of tumor-derived chaperone proteins (often called heat shock proteins, or HSPs) as anti-cancer vaccines has reached clinical trial stages^{48, 49} after numerous reports of the immunizing efficacy of these proteins in animal models⁵⁰. The operational paradigm for the immunological effects of tumor-derived chaperone proteins involves both innate and adaptive responses. The innate responses are derived from the pro-inflammatory (cytokine-like) effects that exogenous, extracellular chaperone proteins have on professional APCs, particularly dendritic cells, presumably due to interactions with APC surface receptors for chaperone proteins^{51, 52, 53, 54, 55, 56}. These effects on DCs include the upregulation co-stimulatory molecules such as CD40, CD80, and CD86, as well as increased expression of MHC class I and II molecules and activation of the NF- κ B pathway^{57, 58, 59, 2}. Also, APCs secrete cytokines such as TNF- α and IL-12 following encounters with chaperone proteins, further enhancing APC stimulation of T cells^{58, 59, 2}. The ensuing adaptive responses are those generated against antigenic peptides that are bound by the chaperone proteins as part of their escort duties. Chaperoned peptides experience "privileged access" to the antigen processing pathway and thus end up being presented to T cells for antigen selection. It is assumed that at least some of the tumor-derived peptides will be antigenic, leading to a T cell anti-tumor response that is enhanced by the chaperone-protein-induced activated state of the APC.

It has been reported that imatinib significantly enhances antigen presentation by bone marrow derived APCs³⁸. We reasoned that in mice treated with combination therapy, both imatinib and CRCL enhanced APC activity, whereby more potent immune responses were generated as demonstrated by the increased IFN- γ production and superior CTL activity. Other formal possibilities may exist to explain the enhanced response; for instance, imatinib may reduce leukemia burden sufficiently to overcome an associated leukemia-induced immunosuppression, which would be evidenced in the increased IFN- γ output by immune effector cells in tumor-bearing mice treated with imatinib and DC/CRCL vaccines. Imatinib may also suppress regulatory T cells that can be envisioned as tolerizing entities, presumably to avoid autoimmune responses. Breaking this tolerance may result in greater activation of T cells that recognize "near self" in the tumor. We are actively pursuing mechanistic answers to the question of how the combination therapy improves treatment outcomes via the immune system.

We should point out that we have not attempted to optimize imatinib therapy in this very aggressive murine leukemia model. The 7-day, 300 mg/kg b.i.d. regimen was chosen to represent essentially a round of chemotherapy and to enable us to observe the effects of adjuvant immunotherapy in conjunction with the drug. Since our scheme with imatinib was very rarely curative outright, we were able to discern the additional benefits of our DC/CRCL vaccines in a therapeutic setting. It is certainly possible that further manipulation of the dosage and duration of imatinib use would improve survival outcomes for drug-treated mice (and therefore, for the combination therapy-treated mice as well). However, the point of this work was to demonstrate the feasibility and efficacy of anti-cancer vaccines in conjunction with an effective drug which in and of itself is not immunosuppressive, and may even be immunosupportive³⁸. The value of these results is in the implication that one may be able to control, if not cure, CML with chemo- and immunotherapeutic combinations.

KEY RESEARCH ACCOMPLISHMENTS

- Immunization of mice with 12B1-derived CRCL loaded DCs induces BCR-ABL specific IFN- γ secretion
- Immunization of mice with 12B1-derived CRCL loaded DCs induces BCR-ABL specific CTL activity
- Immunization of mice with 12B1-derived CRCL loaded DCs provides superior therapeutic effects than DCs loaded with BCR-ABL peptide
- Combination therapy of imatinib and DC/CRCL induces superior IFN- γ secretion and cytotoxic T lymphocyte (CTL) activity in vivo.
- 12B1 cells show in vitro and in vivo sensitivity to imatinib mesylate.
- Imatinib mesylate reduces tumor burden and prolongs survival but does not cure mice of the disease.
- Combination therapy of imatinib mesylate with DCs pulsed with 12B1-derived CRCL can be curative against mice bearing 12B1 tumor.

REPORTABLE OUTCOMES

Manuscripts

1. Zeng Y., Graner M.W., Li G., Feng H., Katsanis E. Imatinib mesylate effectively combines with chaperone-rich cell lysate loaded dendritic cells to treat bcr-abl⁺ murine leukemia. *International Journal of Cancer* (in press)
2. Zeng Y., Graner M.W., Katsanis E. Immunization of mice with bcr-abl⁺ tumor derived chaperone-rich cell lysates induces bcr-abl peptide specific immunity. (in preparation)

Abstracts

1. Zeng Y., Graner M.W., Feng H., Katsanis E. Leukemia-derived chaperone-rich cell lysates (CRCL) provide bcr-abl specific immunity Presented at the American Society of Hematology, San Diego, California December 2003. *Blood* 102:655a, 2003.
2. Zeng Y., Graner M.W., Feng H., Li G., Katsanis E. Imatinib mesylate effectively combines with chaperone-rich cell lysate loaded dendritic cells to treat bcr-abl⁺ murine leukemia. *Blood* 102:322b, 2003.

CONCLUSIONS

In conclusion, using functional immunological assays, we have shown that BCR-ABL peptides are chaperoned by 12B1 tumor derived CRCL and are cross-presented to T cells. In addition, other antigenic peptides may also be present in CRCL antigen repertoire. Future study will include the identification of BCR-ABL peptides, as well as other antigenic peptides in CRCL using biochemical techniques.

Moreover, we feel our data provide compelling evidence for the utilization of chaperone protein-based anti-cancer vaccines in combination with specific chemotherapy, in this case imatinib mesylate. While the search for better drug combinations to pair with imatinib continues, we suggest that immunotherapy be given a higher priority in that endeavor. CRCL vaccines, especially when used as an immunogen for pulsing DCs, may represent a novel form of immunotherapy ideally suited for augmenting the targeted imatinib chemotherapy.

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